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(54) Title: METHODS OF ASSAYING FOR G PROTEIN-COUPLED RECEPTOR LIGANDS AND MODULATORS

(57) Abstract: The present invention features methods of assaying for ligands and/or modulators of G protein-coupled receptors, for example, orphan G protein-coupled receptors. The methods of the invention feature constitutively active arrestin mutants, in particular, phosphorylation independent mutants. Also described are specific phosphorylation independent arrestin mutants and methods of making such mutants.

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METHODS OF ASSAYING FOR G PROTEIN-COUPLED RECEPTOR LIGANDS AND MODULATORS

Related Applications

5 This application claims the benefit of prior-filed provisional patent application U.S. Serial No. 60/186,706, entitled "Methods of Assaying for G Protein-Coupled Receptor Ligands and Modulators", filed March 3, 2000 (pending). The content of the above referenced application is incorporated herein in its entirety by this reference.

10 Background of the Invention

 G protein-coupled receptors (GPCRs) are integral membrane proteins involved in the transmission of signals from the extracellular environment to the cytoplasm (for review see *e.g.*, Gutkind (1998) *J. Biol. Chem.* 273:1839-1842). The best known family of G protein-coupled receptors (GPCRs), currently comprises more than 1000 members and exhibits a
15 common structural motif consisting of seven membrane-spanning regions (Dohlman *et al.* (1987) *Biochemistry* 26:2657-2664). Sixteen distinct mammalian G α protein subunits have been molecularly cloned and are divided into four families based upon sequence similarity: G α_s , G α_i , G α_q , and G α_{12} . Similarly, eleven G β protein subunits and five G protein subunits have been identified. Thus, GPCRs are likely to represent the most diverse signal
20 transduction systems in eukaryotic cells.

 A diverse array of external stimuli including neurotransmitters, hormones, phospholipids, photons, odorants, certain taste ligands, and growth factors can activate members of this receptor family and promote interaction between the receptor and a G protein located on the intracellular side of the membrane. This causes the exchange of GDP
25 for GTP bound to the G protein α subunit and further causes the dissociation of the $\beta\gamma$ heterodimers. In turn, GTP-bound G protein α subunits or $\beta\gamma$ complexes initiate intracellular signaling responses by acting on effector molecules such as adenylate cyclases or phospholipases or directly regulating ion channel or kinase function.

 Signaling by diverse GPCR agonists is believed to be terminated by a uniform two-
30 step mechanism (Freedman and Lefkowitz (1996) *Recent Prog. Horm. Res.* 51:319-353). According to the model, activated receptor is first phosphorylated by a G protein-coupled

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receptor kinase (GRK). An arrestin protein then binds to the activated phosphoreceptor, thereby blocking G protein interaction. Arrestin-receptor complex is subsequently internalized, whereupon receptor is either dephosphorylated and recycled back to the plasma membrane (resensitization) or sorted to lysosomes and destroyed (down-regulation). Thus, the formation of the arrestin-receptor complex appears to be the final step of desensitization and the first step of resensitization and/or receptor down-regulation.

GPCRs are activated by an extremely wide variety of external stimuli and are now believed to play a role in regulating the activity of virtually all eukaryotic cells. In contrast, the repertoire of receptor kinases and arrestins involved in the desensitization of these receptors is rather limited: six GRKs and four arrestins have thus far been found in mammals (reviewed in Freedman and Lefkowitz, *supra*).

Summary of the Invention

The fact that a limited number of receptor kinases and arrestins can regulate desensitization of numerous GPCRs makes these molecules attractive targets for research designed to identify potential modulators of GPCR signaling, in particular modulators of such receptors for use as human therapeutics. The present invention is based, at least in part, on a new use for phosphorylation-independent mutants of arrestin proteins as assay components in screening assays designed to identify ligands and/or modulators of G protein-coupled receptors (GPCRs). In particular, it has been discovered that phosphorylation-independent mutants of arrestin capable of binding GPCRs in a manner independent of the natural requirement for phosphorylation by intracellular receptor kinases. Such phosphorylation-independent mutants of arrestin retain their dependence on agonist for GPCR-binding, however. As such, phosphorylation-independent arrestin mutants are particularly well-suited for *in vitro* assays, for example, in assays that are directed to the identification of natural and surrogate agonists of orphan GPCRs (*e.g.*, of known and/or orphan GPCRs, for example, in orphan GPCR ligand fishing assays). The phosphorylation-independent mutants of arrestin are also particularly well-suited for *in vitro* assays that are directed to the identification of GPCR antagonists and/or agonists.

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In an exemplary embodiment, the present invention features methods of identifying a GPCR ligands which include contacting a composition containing the GPCR (*e.g.*, a GPCR-containing membrane preparation) and a phosphorylation-independent arrestin mutant with a test compound and determining the ability of the test compound to modulate binding of the arrestin mutant to the GPCR, wherein modulation of binding indicates that the test compound is a GPCR ligand. In one embodiment, the GPCRs being assayed are known GPCRs. In another embodiment, the GPCRs being assayed are orphan G protein-coupled receptors. In another embodiment, the compound being identified is a GPCR modulator (*e.g.*, an agonist or an antagonist or an inverse agonist for the GPCR being assayed). In another embodiment, the compound being identified is a natural and/or surrogate ligand for an orphan GPCR being assayed.

In another embodiment, the present invention features methods of identifying G protein-coupled receptor (GPCR) agonists which include contacting a composition containing the GPCR (*e.g.*, a GPCR-containing membrane preparation), a phosphorylation-independent arrestin mutant and a test compound and determining the ability of the test compound to modulate binding of the arrestin mutant to the GPCR, wherein modulation of binding indicates that the test compound is a GPCR agonist.

In another embodiment, the present invention features methods of identifying G protein-coupled receptor (GPCR) antagonists which include contacting a composition containing the GPCR (*e.g.*, a GPCR-containing membrane preparation), a phosphorylation-independent arrestin mutant and a GPCR agonist with a test compound and determining the ability of the test compound to modulate binding of the arrestin mutant to the GPCR, wherein modulation of binding indicates that the test compound is a GPCR antagonist.

The assays of the present invention are also applicable to screening pluralities of test compounds (*e.g.*, a small molecule library of compounds) as well as screening composition which include more than one GPCR or a plurality of GPCRs. Test compounds can be included in aqueous or organic solutions. Arrestin mutants can be conjugated to a detectable marker (*e.g.*, a colorimetric label, a fluorescent label, a radioisotopic or radiolabel, or a reporter protein). Determining the ability of the test compound to modulate binding of the arrestin mutant to the GPCR can include detecting the binding of a labeled mutant to a gpcr (*e.g.*, a GPCR in a membrane preparation). Determining the ability of the test compound to

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modulate binding of the arrestin mutant to the GPCR can include detecting a change in surface plasmon resonance (SPR).

Also featured are phosphorylation-independent arrestin mutants. Exemplary arrestin mutants include β -arrestin mutants (e.g., β -arrestin 1A mutants, β -arrestin 1B mutants and/or β -arrestin 2 mutants), arrestin C-mutants, X-arrestin mutants and/or visual arrestin mutants. Preferred phosphorylation-independent arrestin mutants have a mutation in the phosphorylation recognition region (e.g., have a mutation in an amino acid corresponding to any one of Lys 161, Arg 162, Arg 166, Arg170 and Lys 171 of SEQ ID NO:4). Additional preferred constitutively-active mutants have a truncated C-terminal regulatory region.

Brief Description of the Drawings

Figure 1 is a graphic depiction of the results of direct binding assays using various forms of arrestins and mutant arrestins (β -arrestins and/or visual arrestins) demonstrating that constitutively-active (i.e., phosphorylation-independent) mutants of the present invention bind G protein-coupled receptors (GPCRs) in a phosphorylation-independent manner. *, $p < 0.01$, Student's t test, compared with the binding of corresponding wild type arrestin. *Figure 1A* depicts the binding of arrestins and mutant arrestins to β_2 -adrenergic receptors (β_2 ARs) and *Figure 1B* depicts the binding to light-activated rhodopsin (Rh).

Figure 2 is a graphic representation of the results of agonist affinity shift assays designed to demonstrate the ability of various phosphorylation-independent arrestin mutants to form high agonist affinity complexes (HACs) with both phosphorylated and non-phosphorylated GPCRs. Unphosphorylated (*Figure 2A*) or phosphorylated (*Figure 2B*) β_2 ARs were incubated with [125 I] iodopindolol (IPIN) and the indicated concentration of agonist in the absence (\circ) or presence of 1 μ M β -arrestin (\bullet), β -arrestin-(Arg169 \rightarrow Glu) (\blacktriangle), β -arrestin-(1-382) (\blacksquare), or β -arrestin-(1-393) (\blacklozenge) as described in Example 2. Means \pm S.D. are presented.

Figure 3 is a graphic depiction of the molecular architecture of arrestins and sequence homology of the phosphorylation recognition region. The major functional regions are designated as follows: R1, basic N-terminal regulatory region; R2, acidic C-terminal regulatory region; A, activation-recognition region; P, phosphorylation recognition region; S, secondary binding site region. The residues corresponding to the borders between

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the functional regions are shown below the schematic (numbering as in bovine β -arrestin 1A, GenBank™ Accession No. P17870, SEQ ID NO:1). Sequences of putative phosphorylation-recognition regions of bovine β -arrestin 1A (corresponding to amino acids 155-184 of SEQ ID NO:1), human β -arrestin 1A (corresponding to amino acids 155-184 of
5 SEQ ID NO:2), human β -arrestin 1B (corresponding to amino acids 155-184 of SEQ ID NO:3), human β -arrestin 2 (corresponding to amino acids 156-185 of SEQ ID NO:4), human arrestin C (corresponding to amino acids 151-181 of SEQ ID NO:5), and human X arrestin (corresponding to amino acids 152-181 of SEQ ID NO:6) are indicated below the schematic.

10 *Figure 4A-B* is a Clustal W alignment of the amino acid sequences of the following human arrestin proteins: β -arrestin 1A (GenBank™ Accession No. AAC33295, corresponding to SEQ ID NO:2), β -arrestin 1B (GenBank™ Accession No. AAC34123, corresponding to SEQ ID NO:3), β -arrestin 2 (GenBank™ Accession No. AAC99468, corresponding to SEQ ID NO:4), arrestin C (GenBank™ Accession No. AAC78395,
15 corresponding to SEQ ID NO:5), and X arrestin (GenBank™ Accession No. AAB84302, corresponding to SEQ ID NO:6). The alignment was generated using the Clustal W1.7 algorithm (parameters are as follows: Pairwise Alignment, K-tuple = 1, Window size = 5, Gap Penalty = 3, Number of Top Diagonals = 5; Multiple Alignment Parameters, Gap Open Penalty = 10, Gap Extension Penalty = 0.05, Blosum Matrix). Phosphorylation recognition
20 domains are indicated in bold and C-terminal regulatory domains are indicated in bold italics. Asterisks above the β -arrestin 1A amino acid sequence indicate exemplary residues mutated in phosphorylation-independent arrestin mutants of the present invention. Asterisks and (:) below the X arrestin amino acid sequence indicate conserved amino acids between
25 arrestin proteins.

Detailed Description of the Invention

The present invention features phosphorylation-independent arrestin mutants and, in particular, features use of such mutants in *in vitro* screening assays.

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I. General Overview of Assay

In one embodiment, the present invention features methods of identifying a GPCR ligands which include contacting a composition containing the GPCR (*e.g.*, a GPCR-containing membrane preparation) and a phosphorylation-independent arrestin mutant with a
5 test compound and determining the ability of the test compound to modulate binding of the arrestin mutant to the GPCR, wherein modulation of binding indicates that the test compound is a GPCR ligand. As used herein, the term "contacting" (*i.e.*, contacting a composition comprising a G protein-coupled receptor with a compound or agent) is intended to include incubating the compound or agent and composition together *in vitro* (*e.g.*, adding
10 the compound or agent to the composition in a suitable vessel) such that the compound or agent has the potential, for example, to interact with and/or bind to a GPCR which exists in the composition.

In one embodiment, the GPCRs being assayed are known G protein-coupled receptors and the ligand being identified is an unknown, additional or alternative ligand for
15 the GPCR being assayed. In another embodiment, the GPCRs being assayed are orphan G protein-coupled receptors and the ligand being identified is a natural ligand for the orphan GPCR being assayed or, alternatively, is a surrogate ligand for the orphan GPCR being assayed.

In another embodiment, the present invention features methods of identifying a G
20 protein-coupled receptor (GPCR) agonists which include contacting a composition containing the GPCR (*e.g.*, a GPCR-containing membrane preparation) and a phosphorylation-independent arrestin mutant with a test compound and determining the ability of the test compound to modulate binding of the arrestin mutant to the GPCR, wherein modulation of binding indicates that the test compound is a GPCR agonist.

25 In another embodiment, the present invention features methods of identifying a G protein-coupled receptor (GPCR) antagonists which include contacting a composition containing the GPCR (*e.g.*, a GPCR-containing membrane preparation), a phosphorylation-independent arrestin mutant and a GPCR agonist with a test compound and determining the ability of the test compound to modulate binding of the arrestin mutant to the GPCR,
30 wherein modulation of binding indicates that the test compound is a GPCR antagonist.

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Yet another embodiment of the present invention features a method for identifying a GPCR modulator which includes contacting a composition containing a constitutively-active mutant GPCR (CAM GPCR) and a phosphorylation-independent arrestin mutant with a test compound and determining the ability of the test compound to modulate binding of the

5 arrestin mutant to the CAM GPCR, wherein modulation of binding indicates that the test compound is a GPCR modulator. As defined herein, a "constitutively active mutant GPCR" is a mutant GPCR protein that can activate GPCR-signaling activity in the absence of GPCR ligand or agonist binding. "Constitutively active mutant GPCRs" have basal receptor activity even in the absence of ligand binding. Accordingly, such "constitutively active

10 mutant GPCRs" can be used in conjunction with the phosphorylation-independent mutant arrestins of the present invention is assay methods to identify GPCR modulators (*e.g.*, inverse agonists of said GPCRs), even in the absence of GPCR ligands. In an exemplary embodiment, the invention features a method of identifying an orphan GPCR modulator (*e.g.*, an inverse agonist of an orphan GPCR) which includes contacting an orphan CAM

15 GPCR and a phosphorylation-independent arrestin mutant with a test compound and determining the ability of the test compound to modulate binding of the arrestin mutant to the orphan CAM GPCR, wherein modulation of binding indicates that the test compound is an orphan GPCR modulator.

In another embodiment, the present invention features methods of identifying a

20 GPCR ligands which include contacting a composition containing the GPCR (*e.g.*, a GPCR-containing membrane preparation) and a phosphorylation-independent arrestin mutant with a test compound and determining the ability of the test compound to modulate binding of the arrestin mutant to the GPCR, wherein modulation of binding indicates that the test compound is a GPCR ligand. In one embodiment, the GPCRs being assayed are known

25 GPCRs. In another embodiment, the GPCRs being assayed are orphan G protein-coupled receptors. In another embodiment, the compound being identified is a GPCR modulator (*e.g.*, an agonist or an antagonist or an inverse agonist for the GPCR being assayed). In another embodiment, the compound being identified is a natural and/or surrogate ligand for an orphan GPCR being assayed.

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Determining the ability of a test compound to modulate binding of an arrestin mutant to a GPCR can include detecting a change in the binding of a labelled mutant to the GPCR (*e.g.*, the GPCR included in a membrane preparation). Determining the ability of the test compound to modulate binding of the arrestin mutant to the GPCR can also include
5 detecting a change in surface plasmon resonance (SPR).

The assays of the present invention are applicable to screening pluralities of test compounds (*e.g.*, a small molecule library of compounds) as well as screening composition which include more than one GPCR or a plurality of GPCRs. Test compound can be included in aqueous or organic solutions. Arrestin mutants can be conjugated to a detectable
10 marker (*e.g.*, a colorimetric label, a fluorescent label, a radioisotopic or radiolabel, or a reporter protein).

Also featured are phosphorylation-independent arrestin mutants. Exemplary arrestin mutants include β -arrestin mutants (*e.g.*, β -arrestin 1A mutants, β -arrestin 1B mutants and/or β -arrestin 2 mutants), arrestin C-mutants, X-arrestin mutants and/or visual arrestin mutants.
15 Additional exemplary phosphorylation-independent arrestin mutants have a mutation in the phosphorylation recognition region (*e.g.*, have a mutation in an amino acid corresponding to any one of Lys 161, Arg 162, Arg 166, Arg 170 and Lys 171 of SEQ ID NO:4). Additional preferred phosphorylation-independent arrestin mutants have a truncated C-terminal regulatory region. In an exemplary embodiment, a phosphorylation-independent arrestin
20 mutant has the amino acid sequence set forth as SEQ ID NO:7. In another embodiment, a phosphorylation-independent arrestin mutant has the amino acid sequence of SEQ ID NO:2, lacking about amino acid residues 351-418. In another embodiment, a phosphorylation-independent arrestin mutant has the amino acid sequence set forth as SEQ ID NO:8. In another embodiment, a phosphorylation-independent arrestin mutant has the amino acid
25 sequence of SEQ ID NO:4, lacking about amino acid residues 343-410. In another embodiment, a phosphorylation-independent arrestin mutant has the amino acid sequence of SEQ ID NO:4, lacking about amino acid residues 344-409. In another embodiment, a phosphorylation-independent arrestin mutant has the amino acid sequence of SEQ ID NO:5, lacking about amino acid residues 348-388. In yet another embodiment, a phosphorylation-independent arrestin mutant has the amino acid sequence of SEQ ID NO:6, lacking about
30 amino acid residues 348-388.

II. Assay Formats

The present invention features a variety of assay formats suitable for identification of GPCR ligands and/or modulators. For example, assay components can be interacted or
5 contacted in a reaction vessel suitable for reacting assay components (*e.g.*, in a tube, for example, a test tube or microfuge tube, in a well, for example, in a microtitre-plate well, on a solid surface, for example, in a droplet or microdroplet, on a chip, or the like). In a preferred embodiment, at least one assay component can be immobilized in an assay well or on an assay surface either directly or indirectly and additional assay components interacted
10 or contracted with said immobilized components.

In instances where, for example, it is desirable to utilize labeled or unlabeled arrestin mutants as an assay component or reagent, it may be desirable to immobilize the GPCR (or a GPCR-containing composition) to an assay surface (*e.g.*, an assay well or chip) for example, in high throughput screening (HTS) or BIACORE™ assay formats described in
15 Examples 4 and 5 herein.

Accordingly, in one embodiment, a GPCR or GPCR-containing composition (*e.g.*, a purified and/or isolated GPCR in a suitable buffer or medium) can be immobilized to an assay surface directly, for example, *via* a covalent attachment (*e.g.*, immobilization via native -NH₂, -SH, -CHO and/or COOH groups in the GPCR). In another embodiment, the
20 composition can be immobilized to the assay surface indirectly. For example, an antibody specific for the GPCR can be immobilized on the assay surface (*e.g.*, *via* direct conjugation or *via* an anti-Ig or anti-Fc antibody to the specific GPCR antibody. Alternatively, the GPCR (or GPCR-containing composition) can be chemically modified (*e.g.*, biotinylated) and immobilized, for example, on a streptavidin-coated assay surface.

25 The present invention further features assay formats in which GPCRs are included in membrane-like environment (*e.g.*, membrane preparations or liposome) designed to allow interaction of the GPCRs with other assay components or reagents (*e.g.*, mutant arrestin, and/or test compounds) in, for example, aqueous buffers.

In one embodiment, a GPCR in a membrane-like environment is immobilized to the
30 assay surface directly. For example, a membrane prep (comprising at least one GPCR) can be immobilized *via* direct hydrophobic adsorption to the surface (*e.g.*, *via* hydrophobic

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adsorption to an assay well using a hydrophobic adsorption (HPA) sensor chip). In another embodiment, a GPCR in membrane-like environment (*e.g.*, in a membrane preparation comprising at least one GPCR) is immobilized to the assay surface indirectly. For example, an antibody specific for a target component in the composition can be immobilized on the assay surface *e.g.* via an anti-Fc or anti-IgG antibody to said component-specific antibody followed by immobilization of the GPCR-containing composition *via* interaction of said component-specific antibody with the target component included in the component included in the GPCR-containing composition. As used herein, a "target component" includes a component included, for example, in a membrane preparation to facilitate immobilization of the membrane preparation. Preferred target components include at least one GPCR in the membrane composition or a molecule (*e.g.*, membrane protein) for which a binding partner is known, said binding partner being suitable for direct immobilization to the assay surface (*e.g.*, an antibody specific for the target component, strepavidin, glutathione S-transferase (GST), maltose E binding protein, protein A, or the like). In yet another embodiment, for example, a GPCR in a membrane-like environment, can be immobilized to the assay surface *via* affinity capture of a second component (*e.g.*, a target component) in the membrane preparation or liposome. For example, a known ligand for a first target receptor in the membrane preparation or liposome can be immobilized on the assay surface and used to affinity capture the membrane preparation or liposome, containing another or second receptor (*e.g.*, an orphan GPCR for which identification of a surrogate ligand is sought). The above-described immobilization techniques are also applicable in instances in which it is desirable to utilize unlabeled arrestin mutants and/or unlabeled GPCRs as assay reagents or components, for example, in a BIACORE™ assay format as described herein. (*See e.g.* Example 4.)

In yet other embodiments, it may be desirable to utilize unlabeled arrestin mutants immobilized on an assay surface (*e.g.*, on a biomolecular sensor chip or BIACORE™ chip). Arrestin mutants can be immobilized using any of the direct or indirect immobilization techniques described above. Alternatively, arrestin mutants (or GPCRs, for example, in any of the above-described assay formats) can be immobilized using any other art recognized means for immobilizing proteins to solid surfaces.

III. Assay Reagents

A. Preparation of Mutant Arrestin Proteins

The present invention further features isolated or purified phosphorylation-independent arrestin mutants, or biologically active portions thereof. As used herein, a
5 “phosphorylation-independent arrestin mutant” is an arrestin mutant that binds one or more GPCRs in a phosphorylation independent manner. A “phosphorylation-independent arrestin mutant” can be identified according to any assay designed to test the binding of the mutant to a GPCR. Mutants which have the ability to bind GPCR in the absence of phosphorylation of the GPCR (*e.g.*, phosphorylation of the receptor by a GPCR-kinase or
10 GRK) are said to bind in a “phosphorylation-independent” manner. An exemplary assay in which to test whether a potential phosphorylation-independent arrestin mutant has the ability to bind GPCR in a phosphorylation-independent manner is described in detail in Example 1.

The phrase “mutant arrestin” includes a protein differing in its amino acid sequence from a naturally-occurring arrestin protein such that the mutant arrestin has an altered
15 biological activity as compared to the naturally-occurring arrestin protein. Exemplary “phosphorylation-independent arrestin mutants” include an arrestin proteins having a mutation in their amino acid sequence which results in the arrestin being capable of binding a GPCR in the absence of phosphorylation of the arrestin protein. Additional exemplary “phosphorylation-independent arrestin mutants” include an arrestin protein having a
20 mutation in its amino acid sequence which results in the arrestin protein having a substantially reduced dependence on phosphorylation as a requisite or requirement for GPCR-binding activity.

The phrase “mutant arrestin” is not intended to include arrestin proteins having amino acid substitutions, insertions and/or deletions which do not alter a biological activity
25 of the protein. According to the present invention, arrestin protein having insertions, substitutions and/or deletions which do not affect or alter a biological activity of an arrestin can be termed “variants” or “homologues”. As such, the present invention is intended to include mutants (*e.g.*, phosphorylation-independent mutants) of the arrestins set forth, for example, as SEQ ID NOs:2-5, as well as phosphorylation-independent mutants or other
30 arrestin homologues and/or variants.

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In one embodiment, an phosphorylation-independent arrestin mutant has an amino acid sequence at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95% or more homologous or identical to any of the amino acid sequences set forth as SEQ ID NO.:2-5 and has a mutation in said amino acid sequence such that the mutant binds GPCRs in a phosphorylation-independent manner. To determine the percent homology of two amino acid sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in the sequence of a first amino acid for optimal alignment with a second amino or nucleic acid sequence and non-homologous sequences can be disregarded for comparison purposes). In a preferred embodiment, the length of a reference sequence aligned for comparison purposes is at least 30%, preferably at least 40%, more preferably at least 50%, even more preferably at least 60%, and even more preferably at least 70%, 80%, or 90% of the length of the reference sequence. The amino acid residues at corresponding amino acid positions are then compared. When a position in the first sequence is occupied by the same amino acid residue as the corresponding position in the second sequence, then the molecules are homologous at that position (i.e., as used herein amino acid or nucleic acid "homology" is equivalent to amino acid or nucleic acid "identity"). The percent homology between the two sequences is a function of the number of identical positions shared by the sequences (i.e., % homology = # of identical positions/total # of positions x 100).

The comparison of sequences and determination of percent homology between two sequences can be accomplished using a mathematical algorithm. A preferred, non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Karlin and Altschul (1990) Proc. Natl. Acad. Sci. USA 87:2264-68, modified as in Karlin and Altschul (1993) Proc. Natl. Acad. Sci. USA 90:5873-77. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al., (1997) Nucleic Acids Research 25(17):3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used. See <http://www.ncbi.nlm.nih.gov>.

Another preferred, non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller (1988) *Comput. Appl. Biosci.* 4:11-17. Such an algorithm is incorporated into the ALIGN program available, for example, at the GENESTREAM network server, IGH Montpellier, FRANCE (<http://vega.igh.cnrs.fr>)

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or at the ISREC server (<http://www.ch.embnet.org>). When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used.

For example, arrestin mutant proteins having amino acid substitutions at "non-essential" amino acid residues can be used according to the present invention. A "non-essential" amino acid residue is a residue that can be altered from the wild-type sequence of the exemplary arrestin mutant proteins described herein without altering the functional activity, whereas an "essential" amino acid residue is required for biological activity. For example, amino acid residues that are conserved among the arrestin mutant proteins of the present invention, are predicted to be particularly unamenable to alteration. Such conserved amino acids can be determined, at least for example, from an alignment of the amino acid sequences of the exemplary arrestin mutant proteins described herein.

A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine).

Exemplary phosphorylation-independent arrestin mutants have a mutation in the phosphorylation recognition domain of the protein. As used herein, a "phosphorylation recognition domain" includes an arrestin protein domain having about 25-25, 26-34, 27-33, 28-32, 29-31 or 30 amino acid residues and is involved in recognition of phosphorylated GPCR by an arrestin protein. A phosphorylation recognition includes basic residues which are involved in recognition of phosphorylated receptor by the arrestin protein. In one embodiment, a "phosphorylation recognition domain" includes about amino acid residues 155-184 of SEQ ID NO:2. In another embodiment, a "phosphorylation recognition domain" includes about amino acid residues 155-184 of SEQ ID NO:3. In another embodiment, a "phosphorylation recognition domain" includes about amino acid residues 156-185 of SEQ

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ID NO:4. In another embodiment, a “phosphorylation recognition domain” includes about amino acid residues 151-181 of SEQ ID NO:5. In another embodiment, a “phosphorylation recognition domain” includes about amino acid residues 152-181 of SEQ ID NO:6. Mutants within a “phosphorylation recognition domain” can include mutations of at least one basic residue involved in phosphoamino acid recognition of phosphorylated residues in a GPCR. Exemplary basic residues which can be mutated include lysines and/or arginines. In one embodiment, at least one amino acid residue selected from the group consisting of Lys 161, Arg 162, Arg 166, Arg170 and Lys 171 of SEQ ID NO:4 is mutated. In another embodiment, a corresponding amino acid in an arrestin protein other than that set forth as SEQ ID NO:4 (*e.g.*, an amino acid corresponding to an amino acid residue selected from the group consisting of Lys 161, Arg 162, Arg 166, Arg170 and Lys 171 of SEQ ID NO:4) is mutated in said other arrestin protein. Amino acid residues, for example, corresponding to an amino acid residue selected from the group consisting of Lys 161, Arg 162, Arg 166, Arg170 and Lys 171 of SEQ ID NO:4, are set forth in Figure 5. “Corresponding” amino acid residues in any other arrestin protein can be determined by optimally aligning the other arrestin to any one of the arrestin proteins set forth as SEQ ID NO:s 2-6 (*e.g.*, the protein set forth as SEQ ID NO:4) and visualizing basic amino acid residues within the other arrestin protein which align, for example, an amino acid residue selected from the group consisting of Lys 161, Arg 162, Arg 166, Arg170 and Lys 171 of SEQ ID NO:4.

Exemplary phosphorylation-independent arrestin mutants alternatively or further have a truncation of a C-terminal regulatory domain of the protein. As used herein, a “C-terminal regulatory domain” includes an arrestin protein domain having about 40-70, preferably about 40-45, 45-50, 55-60 or 65-70 amino acid residues and serves a regulatory role in controlling arrestin binding to a phosphorylated (and/or) activated form of a GPCR without directly participating in the receptor interaction. In one embodiment, a C-terminally truncated arrestin mutant lacks at least about 10-20 C-terminal amino acid residues. In another embodiment, a C-terminally truncated arrestin mutant lacks at least about 20-30 C-terminal amino acid residues. In another embodiment, a C-terminally truncated arrestin mutant lacks at least about 30-40, 40-50, 50-60, 60-70, 70-80 or more C-terminal amino acid residues. Exemplary C-terminally truncated arrestin mutants include the amino acid sequence of SEQ ID NO:2, lacking about amino acid residues 351-418, the amino acid

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sequence of SEQ ID NO:4, lacking about amino acid residues 343-410, the amino acid sequence of SEQ ID NO:4, lacking about amino acid residues 344-409, the amino acid sequence of SEQ ID NO:5, lacking about amino acid residues 348-388, or the amino acid sequence of SEQ ID NO:6, lacking about amino acid residues 348-388.

5 In other exemplary embodiments, an arrestin mutant protein of the present invention is a mutant of a human arrestin protein selected from the group consisting of human β -arrestin 1A (GenBank™ Accession No. AAC33295, corresponding to SEQ ID NO:2), human β -arrestin 1B (GenBank™ Accession No. AAC34123, corresponding to SEQ ID NO:3), human β -arrestin 2 (GenBank™ Accession No. AAC99468, corresponding to SEQ ID NO:4), human arrestin C (GenBank™ Accession No. AAC78395, corresponding to SEQ ID NO:5), and human X arrestin (GenBank™ Accession No. AAB84302, corresponding to SEQ ID NO:6), said mutant having either a mutation within the phosphorylation recognition domain or having a truncation of all or a part of the C-terminal regulatory domain.

As used herein, an "isolated" or "purified" arrestin mutant, or biologically active portion thereof, is substantially free of cellular material or other contaminating proteins from the cell or tissue source from which the mutant is derived, or substantially free from chemical precursors or other chemicals when chemically synthesized. The language "substantially free of cellular material" includes preparations of arrestin mutant in which the protein is separated from cellular components of the cells from which it is isolated or recombiantly produced. In one embodiment, the language "substantially free of cellular material" includes preparations of arrestin mutant having less than about 30% (by dry weight) of non-arrestin mutant protein (also referred to herein as a "contaminating protein"), more preferably less than about 20% of non-arrestin mutant protein, still more preferably less than about 10% of non-arrestin mutant protein, and most preferably less than about 5% non-arrestin mutant protein. When the arrestin mutant or biologically active portion thereof is recombiantly produced, it is also preferably substantially free of culture medium, *i.e.*, culture medium represents less than about 20%, more preferably less than about 10%, and most preferably less than about 5% of the volume of the mutant preparation.

The language "substantially free of chemical precursors or other chemicals" includes preparations of arrestin mutant protein in which the protein is separated from chemical precursors or other chemicals which are involved in the synthesis of the protein. In one

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embodiment, the language “substantially free of chemical precursors or other chemicals” includes preparations of arrestin mutant protein having less than about 30% (by dry weight) of chemical precursors, more preferably less than about 20% chemical precursors, still more preferably less than about 10% chemical precursors, and most preferably less than about 5% chemical precursors.

As used herein, a “biologically active portion” of an arrestin mutant protein includes a fragment of an arrestin mutant which participates in an interaction between the mutant protein molecule and, for example, a GPCR. Biologically active portions of arrestin mutants protein include peptides comprising amino acid sequences sufficiently identical to or derived from the amino acid sequence of the arrestin mutant, *e.g.*, the amino acid sequence shown in SEQ ID NO:7 of SEQ ID NO:8, which include less amino acids than the full length arrestin mutant proteins, and exhibit at least one activity of an arrestin mutant protein. Biologically active portions of an arrestin mutant protein can be substituted for full-length arrestin proteins in the screening assays of the present invention, *e.g.*, in screening assays for agents which bind GPCRs.

Arrestin mutant proteins of the present invention can be made according to recombinant techniques. In general, recombinant production of arrestin mutant protein can be accomplished by inserting the coding sequence of the gene encoding a arrestin mutant protein into a recombinant expression vector in the context of the appropriate regulatory elements, such that expression of the recombinant arrestin mutant protein is achieved. The arrestin mutant proteins further can be purified from cells. Such cells include, but are not limited to, bacterial cells, yeast cells, fungal cells and mammalian cells. Such a procedure is described in detail, for example, in Example 1. Purification of arrestin mutant proteins from cells can be accomplished according to the procedures described herein for the isolation of arrestin mutant proteins or, alternatively, can be purified from appropriate biological sources.

Isolated nucleic acid molecules which encode the phosphorylation-independent mutants of the present invention are also within the scope of the invention. The term “isolated nucleic acid molecule” includes nucleic acid molecules which are substantially free of other cellular material when purified, for example, from biological samples (*e.g.*, tissue samples), or substantially free of culture medium when produced by recombinant

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techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized. Moreover, an "isolated" nucleic acid molecule, such as a cDNA molecule, can be one which is separated from other nucleic acid molecules which are present in the natural source of the nucleic acid. For example, an "isolated" nucleic acid molecule, such as a
5 cDNA molecule, can be one which is separated from the chromosome with which the corresponding genomic DNA is naturally associated. Preferably, such an "isolated" nucleic acid is free of sequences which naturally flank the nucleic acid (*i.e.*, sequences located at the 5' and 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived.

10 Also included within the scope of the invention are such phosphorylation-independent mutant-encoding nucleic acid molecules included within a vector (*e.g.*, included within an expression vector) as well as such vectors included within a host cell.

B. Preparation of GPCR Proteins

15 GPCR proteins of the present invention can also be produced using recombinant protein expression technologies. For example, the GPCRs of the present invention can be produce in organisms including bacteria (*e.g.*, *Escherichia coli*) yeast (*e.g.*, *Saccharomyces cerevisiae* or *Candida parapsilosis*) and/or mammalian cells. Production of GPCRs in *E. coli* can be accomplished with slight modification of standard recombinant expression
20 procedures *e.g.*, those used to recombinantly produce globular proteins) taking into account the fact that GPCRs are integral membrane proteins. For example, the anionic detergent, sarkosyl, can be utilized to enable the extraction of the expressed GPCRs from inclusion bodies (when GPCRs are produced in *E. coli*) in a form that can be functionally reconstituted into membrane preparations.

25

C. Membrane Preparations

Membrane preparations, for example, receptor-containing preparations (*e.g.*, preparations containing GPCRs), can be prepared according to any art recognized methodologies of membrane preparation (*e.g.*, according to Burgisser et al. (1985) *Biochem. Biophys. Res. Commun.* 133:1202-1209). As used herein, the term "membrane preparation" includes. In
30 general, processes for preparing membrane preparations involves the obtaining of suitable

cellular or tissue material (*e.g.*, eukaryotic tissues or cell cultures, for example, cultures cells expressing or overexpressing GPCRs, in particular, orphan GPCRs). This is followed by cellular disintegration, for example, by homogenization, ultrasonics, detergent or organic solvent lysis, agitation, high temperature or some other suitable procedure, followed by
5 separation of cytoplasmatic components (*e.g.*, soluble components) and/or separation of coarser particles (*e.g.*, by centrifuging, filtering, and the like). In an exemplary embodiment, membrane preparations of the present invention can be prepared according to the methodologies described in "Reconstitution of receptor-promoted signal transduction using purified receptor, G protein, and effector" Bernstein and Biddlecome (2000)
10 in "G protein coupled receptors", eds. Haga and Bernstein, pp. 117-154, CRC Press LLC, Boca Raton, FL.

Alternatively, membrane preparations for use in the assays of the present invention can be reconstituted from, for example, membranes prepared according to any of the above-described technologies and purified or isolated GPCRs. A general GPCR purification
15 scheme can involve solubilization of GPCRs with a non-ionic detergent, chromatography on hydroxylapatite, followed in some cases, by SH-affinity chromatography. (See, for example, Wohlrab (1986) *Biochim. Biophys. Acta* 853:8170-8173; Wehrle and Pedersen (1989) *J. Membr. Biol.* 111:199-213; Kramer. and Palmeri (1989) *Biochim. Biophys. Acta* 974:1-23; Kolbe *et al.* (1984) *J. Biol. Chem.* 259:9115-9120; Kaplan *et al.* (1986) *J. Biol. Chem.* 261:12767-12773; and Guerin *et al.* (1990) *J. Biol. Chem.* 265: 19736-19741). An
20 alternative GPCR purification scheme involves solubilization of GPCRs with a non-ionic detergent, chromatography on hydroxylapatite, followed by sequential chromatography on Matrex Gel Orange A™, Matrix Gel Blue B™, and Affi-Gel 501™. (See, for example, Kaplan *et al.* (1990) *J. Biol. Chem.* 265:13379-13385.)

25 Examples of such non-ionic detergent solubilizing agents include digitonin, n-octylglucoside, n-dodecylglucoside, n-dodecylmaltoside, octanoyl-N-methylglucamide, decanoyl-N-methylglucamide, Triton® X-100, Triton® X-114, Thesit®, Isotridecypoly(ethylene glycol ether), 3-[(3-cholamidopropyl)dimethylamminio]-1-propane sulfonate (CHAPS), 3-[(3-cholamidopropyl)dimethylamminio]-2-hydroxy-1-propane
30 sulfonate (CHAPSO), or N-dodecyl=N,N-dimethyl-3-ammonio-1-propane sulfonate.

An exemplary protocol for preparing membranes from cells expressing

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recombinant GPCRs is as follows: Solutions: a. Solution A: 20 mM Tris-Cl (pH 8), 1 mM EDTA, 1 mM EGTA, and PI (protease inhibitors: 0.1 phenylmethylsulfonylfluoride, 2 microg/ml aprotinin, and 10 microg/ml leupeptin); b. Solution B: 20 mM Tris-Cl (pH 8), 3 mM MgCl₂, 10 microg/ml deoxyribonuclease I, and PI. 1. Harvest cells by centrifugation
5 at 1000 xg for 10 min. Discard supernatant. 2. Resuspend cell pellet with ice-cold solution A. 3. Homogenize the cell suspension with a Dounce homogenizer, 10 strokes. 4. Centrifuge homogenate at 30,000 xg for 20 min. Discard supernatant. 5. Repeat steps 3. and 4. with the pellet. 6. Resuspend pellet (membrane fraction) with a small volume of solution B with a Dounce homogenizer. 7. Aliquot as needed and store at -80 degree C
10 until use.

D. Labeled reactants

Exemplary labels for labeling reactants for use in the assay methods of the present invention include radiolabels (*e.g.*, radioactive isotopes including but not limited to ³⁵S, ¹⁴C,
15 ³H, ³²P and ¹²⁵I), colorimetric labels (*e.g.*, chromophores), reporter proteins and fluorescent labels (*e.g.*, fluorophores). Such labels as well as methods of incorporating such labels are known in the art.

E. Test Compounds

20 The test compounds of the present invention can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the 'one-bead one-compound' library method; and synthetic library methods using affinity chromatography selection. The
25 biological library approach is limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam, K.S. (1997) *Anticancer Drug Des.* 12:145).

Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt et al. (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90:6909; Erb et al.
30 (1994) *Proc. Natl. Acad. Sci. USA* 91:11422; Zuckermann et al. (1994). *J. Med. Chem.* 37:2678; Cho et al. (1993) *Science* 261:1303; Carrell et al. (1994) *Angew. Chem. Int. Ed.*

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Engl. 33:2059; Carell et al. (1994) *Angew. Chem. Int. Ed. Engl.* 33:2061; and in Gallop et al. (1994) *J. Med. Chem.* 37:1233.

Libraries of compounds may be presented in solution (*e.g.*, Houghten (1992) *Biotechniques* 13:412-421), or on beads (Lam (1991) *Nature* 354:82-84), chips (Fodor (1993) *Nature* 364:555-556), bacteria (Ladner USP 5,223,409), spores (Ladner USP '409), plasmids (Cull et al. (1992) *Proc Natl Acad Sci USA* 89:1865-1869) or on phage (Scott and Smith (1990) *Science* 249:386-390); (Devlin (1990) *Science* 249:404-406); (Cwirla et al. (1990) *Proc. Natl. Acad. Sci.* 87:6378-6382); (Felici (1991) *J. Mol. Biol.* 222:301-310); (Ladner *supra.*).

Exemplary compounds which can be screened for activity include, but are not limited to, peptides, nucleic acids, carbohydrates, small organic molecules, and peptidomimetics. The term "peptidomimetic" is intended to encompass compounds that are comprised, at least in part, of molecular structures different from naturally-occurring L-amino acid residues linked by natural peptide bonds. "Peptidomimetics" are intended to include compounds composed, in whole or in part, of structures such as D-amino acids, non-naturally-occurring L-amino acids, modified peptide backbones and the like, as well as compounds that are composed, in whole or in part, of molecular structures unrelated to naturally-occurring L-amino acid residues linked by natural peptide bonds.

F. Automated Screening Methods

The methods of the present invention may be automated to provide convenient, real time, high volume methods of screening test compounds for the ability to bind to or modulate GPCRs (*e.g.*, screening for surrogate ligands of orphan GPCRs or modulators of GPCRs). Automated methods can be optimized to detect binding of labeled assay components (*e.g.*, labeled arrestin mutant protein) to GPCR, optionally in the presence or absence of known GPCR agonist, or to detect, for example, binding of unlabeled assay components, optionally in the presence or absence of known GPCR agonist. The binding of assay components can be detected over time (*i.e.*, comparing an assay reaction mixture before and after contacting with a test compound or plurality of test compounds), or by comparison of a test compound or mixture of test compounds to a control assay reaction mixture which is not exposed to the test compound(s), or by comparison to pre-established

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indicia. Both qualitative assessments (positive/negative) and quantitative assessments (comparative levels of binding) may be provided by the present automated methods, as will be apparent to those skilled in the art. The present invention thus further features methods and/or apparatus for automated screening of GPCR ligands (*e.g.*, surrogate ligand

5 identification) or modulators, by detecting the binding of arrestin mutant protein to GPCR in response to test compound(s), optionally in the presence or absence of GPCR agonist.

Arrestin binding may be indicated by an alteration in the distribution of a detectable signal within an assay reaction mixture over time, between a test reaction mixture and a control reaction mixture, or by comparison to previously established parameters. Measurement

10 points may be over time, or among test and control samples or mixtures.

A computer program may be used to process samples, record output, and/or process data obtained by carrying out the above-described steps. The preferred computer program product comprises a computer readable storage medium having computer-readable program code means embodied in the medium. Hardware suitable for use in such automated

15 apparatus will be apparent to those of skill in the art, and may include computer controllers, automated sample handlers, surface plasmon resonance (SPR) measurement tools (*e.g.*, BIACORE™ instruments), tools for measuring bound and/or free labeled reactants, printers and optical displays. The hardware may also contain a computer-controlled stepper motor so that each control and/or test sample can be arranged as an array of samples and

20 automatically and repeatedly positioned for detection of binding of reagents. The measurement tool is preferably operatively coupled to a general purpose or application specific computer controller. The hardware may also comprise a computer program product for controlling operation of the measurement tool and performing numerical operations relating to the above-described steps. For example, a controller may accept set-up and other

25 related data *via* a file, disk input or data bus. A display and printer may also be provided to visually display the operations performed by the controller. It will be understood by those having skill in the art that the functions performed by the controller may be realized in whole or in part as software modules running on a general purpose computer system.

Alternatively, a dedicated stand-alone system with application specific integrated circuits for

30 performing the above described functions and operations may be provided. As provided above, detection of binding of assay reagents may take the form detection of binding of

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labeled reactants or unlabeled reactants, although those skilled in the art will appreciate that other indicia are known and may be used in the practice of the present invention, such as may be provided by labels that produce signals detectable by, for example, X-ray diffraction or absorption or magnetism. Such labels include, for example, electron-dense reagents.

5

G. Cell-Based Assays and Labeled Reactants

Another aspect of the invention features cell-based assays for identifying reagents (e.g., ligands, surrogate ligands, agonists, antagonists and the like) which bind to and/or modulate the activity of GPCRs. In one embodiment, the invention features a method of
10 identifying compounds which bind to and/or modulate the activity of a GPCR that involves contacting a cell which expresses a GPCR and an arrestin mutant of the present invention (e.g., a mutant that interacts with the GPCR in a phosphorylation independent manner) with a test compound and determining the effect of the compound on the interaction between the GPCR and the arrestin mutant, such that a reagent that binds to and/or modulates the activity
15 of the GPCR is identified. Preferably, the interaction between GPCR and arrestin mutant is detectable by labeling one or both of the GPCR and arrestin mutant. In an exemplary embodiment, the interaction between GPCR and arrestin mutant is detectable by labeling the GPCR and/or arrestin mutant with labels detectable as a result of their interaction (e.g., label:label interaction) preferably reagents designed for detecting protein:protein
20 interactions in live cells. For example, bioluminescence resonance energy transfer (BRET) can be used to determine the interaction between GPCR and arrestin mutant. In a preferred embodiment, the arrestin mutant (or a biologically active portion thereof) is recombinantly fused to a BRET donor (e.g., Renilla luciferase) or acceptor (e.g., green fluorescent protein) and energy transfer between labeled arrestin mutant and labeled GPCR (e.g., labeled with
25 acceptor or donor, respectively) is detected. Such a technology is described, for example, in Angers *et al.* (2000) *Proc. Natl. Acad. Sci.* 97:3684-3689, the content of which is incorporated by this reference. In another exemplary embodiment, the GPCR and/or arrestin mutant can be labeled with inactive, weakly-complementing β -galactosidase mutants according to Intercistronic Complementation Analysis Screening Technology
30 ("ICAST"). Association between the molecules of interest (e.g., GPCR and arrestin mutant) brings the complementing β -galactosidase mutants into proximity so that complementation

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occurs and active β -galactosidase is produced. The active β -galactosidase may be detected by methods well-known in the art. Such a technology is described in, for example, WO 98/44350, the content of which is incorporated by this reference. The ICAST technology is also suitable for detection of arrestin mutant:GPCR interaction *in vitro* (e.g., in the cell-free
5 assays described herein).

Preferably, in the cell-based assays (as well as *in vitro* assays) described herein, determining the effectiveness of a test compound is performed in the presence or absence of the test compound, in the presence of the test compound as compared to a suitable control (e.g., a buffer or solvent control, known positive or negative control compounds) or in the
10 presence of the test compound as compared to a predetermined or known range or value for binding and/or activity. Moreover, cell-based agonist assays are preferably performed in the presence of a known ligand specific for the GPCR of interest or, optionally, a surrogate ligand for the GPCR of interest.

15

IV. Preparation and Administration of Pharmaceutical Compositions of GPCR Ligands and/or Modulators

A. Pharmaceutical Preparations of Identified Compounds

20 After identifying certain test compounds as potential modulators of GPCR activity, the practitioner of the subject assay will continue to test the efficacy and specificity of the selected compounds both *in vitro* and *in vivo* as described further herein. Whether for subsequent *in vivo* testing, or for administration to an animal as an approved drug, compounds identified in the subject assay, or a pharmaceutically acceptable salt thereof, can
25 be formulated in pharmaceutical preparations for *in vivo* administration to an animal, preferably a human. Such preparations comprise a therapeutically (or prophylactically) effective amount of the GPCR modulator or ligand, and a pharmaceutically acceptable carrier or excipient. The preparations may be formulated for administration with a pharmaceutically acceptable carrier except insofar as any conventional media or agent is
30 incompatible with the activity of the compound. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents or wetting agents, emulsifying or dispersion

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media, and the like which may be appropriate for the desired route of administration of the pharmaceutical preparation. The composition can be a liquid solution, suspension, emulsion, tablet, pill, capsule, sustained release formulation, or powder. The composition can be formulated as a suppository, with traditional binders and carriers such as

5 triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, *etc.* Other carriers include water, saline, saline buffered with phosphate or citrate at suitable concentrations so as to obtain at all times isotonic pharmaceutical preparations of the desired pH, (for example, neutral pH), dextrose, ethanol, mannitol, polyol (for example,

10 glycerol, propylene glycol, liquid polyethylene glycol and the like) or suitable mixtures thereof. The use of other such media for pharmaceutically active substances is known in the art. The optimum concentration of the active ingredient(s) in the chosen medium can be determined empirically, according to procedures well known to medicinal chemists. The carrier and composition can be sterile. The formulation should suit the mode of

15 administration.

B. Effective Doses of Identified Compounds for Administration

Single and multiple (e.g., 5 to 7 days) dose investigative toxicology studies are typically performed in the efficacy test species using the intended route of administration for

20 the efficacy study. These investigative toxicology studies are performed to identify maximum tolerated dose, subjective bioavailability from the intraperitoneal or oral routes of administration, and estimation of an initial safety margin. Initial bioavailability and pharmacokinetics (blood clearance) of the compounds may be determined, with standard cold or radioactive assay methods, to assist in defining appropriate dosing regimens for the

25 compounds in the animal models.

The effective dose of the GPCR modulator or ligand will typically be in the range of about 0.01 to about 50 mg/kgs, preferably about 0.1 to about 10 mg/kg of mammalian body weight, administered in single or multiple doses. Generally, the GPCR modulator or ligand may be administered to patients in need of such treatment in a daily dose range of about 0.5

30 to about 2000 mg per patient.

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The amount of GPCR modulator or ligand which will be effective in the treatment or prevention of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. In addition, *in vitro* or *in vivo* assays may optionally be employed to help identify optimal dosage ranges. Effective
5 doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems. The precise dosage level of GPCR modulator or ligand, as the active component(s), should be determined by the attending physician or other health care provider and will depend upon well known factors, including route of administration, and the age, body weight, sex and general health of the individual; the nature, severity and clinical stage
10 of the disease; and the use (or not) of concomitant therapies.

Patients in need of treatment with a GPCR modulator include patients having Retinitis pigmentosa, stationary night blindness, color blindness, nephrogenic DI, isolated glucocorticoid deficient, hyperfunctioning thyroid adenomas, familial hypocalciuric hypercalcemia, hyperparathyroidism, neurological disorders and the like.

15

C. Administration of Identified Compounds

Various delivery vehicles are known and can be used to administer the GPCR modulator or ligand, *e.g.*, encapsulation in liposomes, microparticles, injectable "deposit formulations", microcapsules, *etc.* Materials and methods for producing the various
20 formulations are well known in the art (see *e.g.* US Patent Nos. 5,182,293 and 4,837,311 (tablets, capsules and other oral formulations as well as intravenous formulations)). Suitable vehicles and their formulation inclusive of other proteins are described, for example, in the book *Remington's Pharmaceutical Sciences* (Remington's Pharmaceutical Sciences. Mack Publishing Company, Easton, Pa., USA 1985). Administration can be systemic or local.
25 Methods of introduction include but are not limited to pulmonary administration, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural and oral routes, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucosa, rectal and intestinal mucosa, *etc.*) and may be administered together with other biologically active agents. Typically, compositions for
30 intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic to ease pain at

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the side of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed
5 with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration. Topical administration to an individual of an effective amount of the biased agonist can be accomplished by administering the compound(s) directly to the affected area of the skin of
10 the individual. For this purpose, the GPCR modulator or ligand is administered or applied in a composition including a pharmacologically acceptable topical carrier, such as a gel, an ointment, a lotion, or a cream, which includes, without limitation, such carriers as water, glycerol, alcohol, propylene glycol, fatty alcohols, triglycerides, fatty acid esters, or mineral oils.

15 Other topical carriers include liquid petroleum, isopropyl palmitate, polyethylene glycol, ethanol (95%), polyoxyethylene monolaurate (5%) in water, or sodium lauryl sulfate (5%) in water. Other materials such as anti-oxidants, humectants, viscosity stabilizers, and similar agents may be added as necessary.

In cases where it is desirable to administer the GPCR modulator or ligand locally to
20 the area in need of treatment this may be achieved by, for example, and not by way of limitation, local infusion during surgery, topical application, by injection, by means of a catheter, by means of a suppository, or by means of a skin patch or implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers which release the compound into the skin, by either passive or
25 active release mechanisms.

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This invention is further illustrated by the following examples which should not be construed as limiting. The contents of all patents, published patent applications and references cited throughout this application, including Kovoov *et al.* (1999) *J. Biol. Chem.* 274:6831-6834, are incorporated herein by reference. The Figures and the Sequence Listing
5 provided herewith are also incorporated herein by reference.

Examples

Examples 1: Generation and Biological Characterization of Phosphorylation-

5 Independent Arrestin Mutants

Example 1 describes the generation of various phosphorylation-independent mutants and characterization of the biological activity of said mutants *in vitro*. The following materials and methods were used in carrying out the experiments in Example 1 and are
10 further applicable to the methodologies described, for example, in Examples 2-3 described below, as well as in the identification of additional phosphorylation-independent mutants.

Mutagenesis and Biochemical Characterization of β -Arrestins-- Mutations Arg169 \rightarrow Glu (CGG \rightarrow GAG), Gln394 \rightarrow Ter (CAA \rightarrow TAA), and Asp383 \rightarrow Ter (GAT \rightarrow TAG)
15 were introduced by polymerase chain reaction in β -arrestin construct pBARR (Gurevich *et al.* (1995) *J. Biol. Chem.* 270:720-731), that was used for *in vitro* transcription and translation, as described (Gurevich *et al.* (1995), *supra*). NcoI/HindIII 1404-base pair open reading frame was then subcloned into appropriately digested Escherichia coli expression
20 vector pTrcHisB (Invitrogen). All β -arrestin species were expressed in the *in vitro* translation system and tested in the direct binding assay (Gurevich *et al.* (1995), *supra*), overexpressed in *E. coli*, purified to apparent homogeneity (Gray-Keller *et al.* (1997) *Biochemistry* 36:7058-7063), and characterized in the agonist affinity shift assay (Gurevich *et al.* (1997) *J. Biol. Chem.* 272:28849-28852), essentially as described.

Direct Binding Assay-- *In vitro* translated tritiated arrestins (50 fmol) were incubated
25 in 50 mM Tris-HCl, pH 7.5, 0.5 mM MgCl₂, 1.5 mM dithiothreitol, 50 mM potassium acetate with 7.5 pmol of the various functional forms of rhodopsin or with P- β_2 AR or β_2 AR (100 fmol/assay) in a final volume of 50 μ l for 5 min at 37 °C in room light (rhodopsin) or for 60 min at 30 °C in the presence of 0.1 mM -agonist isoproterenol. The samples were immediately cooled on ice and loaded onto 2 ml Sepharose 2B columns equilibrated with 20
30 mM Tris-HCl, pH 7.5, 2 mM EDTA. Bound arrestin eluted with receptor-containing

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membranes in the void volume (between 0.5 and 1.1 ml). Nonspecific binding determined in the presence of 0.3 μ g of liposomes was subtracted.

Agonist Affinity Shift Assay-- P- β_2 AR or β_2 AR (10-15 fmol/assay) was incubated in 0.25 ml of 10 mM Tris-HCl, pH 7.4, 100 mM NaCl (buffer A) containing 0.1 mg/ml bovine
5 serum albumin in the presence of 65-75 fmol of [125 I] iodopindolol (NEN Life Science Products) and the indicated concentrations of arrestins and agonists for 60 min at 22 °C. Samples were then cooled on ice and loaded at 4 °C onto 2 ml of Sephadex G-50 columns. Receptor-containing liposomes with bound radioligand were eluted with buffer A (between 0.6 and 1.5 ml), and radioactivity was quantitated in a liquid scintillation counter.
10 Nonspecific binding was determined in the presence of 10 μ M alprenolol. All experiments were repeated two to three times, and data are presented as means \pm S.D.

It is now understood that arrestin proteins play a key role in the desensitization of GPCRs. It also is now appreciated that naturally-occurring arrestins exhibit selectivity for
15 the activated phosphorylated forms of GPCRs (see *e.g.*, Gurevich and Benovic (1993) *J. Biol. Chem.* 268:11628-11638; Gurevich *et al.* (1995) *J. Biol. Chem.* 270:720-731; and Gurevich *et al.* (1994) *J. Biol. Chem.* 270:720-731). In particular, results of *in vitro* studies indicate that the arrestins have two primary binding sites for GPCRs: an activation-
20 phosphorylation-recognition site or region that recognizes the agonist-activated state of the receptor and a GRK-phosphorylated elements) of the receptor. A potent secondary receptor-binding site is mobilized for the interaction only when both primary sites are simultaneously engaged, *i.e.* when an arrestin encounters activated and phosphorylated receptor. According to this model of arrestin:GPCR interaction, arrestin is kept in its basal conformation by several
25 intramolecular interactions in which certain residues in the primary binding sites ("trigger" residues) are involved. One of these triggers is pulled by binding of the arrestin to an activated form of the receptor, the other, by the interaction of arrestin with phosphate(s) introduced by GRK, for example.

In order to confirm the validity of the model, three bovine β -arrestin mutants were
30 constructed: 1) Arg169 \rightarrow Glu, that reverses the charge of the putative phosphorylation-sensitive trigger (a similar mutation in visual arrestin is described, for example, is Gurevich

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and Benovic (1995) *J. Biol. Chem.* 270:6010-6016); 2) Gln394 → Ter; and 3) Asp383 → Ter, that delete a part or all of the regulatory arrestin COOH terminus, which keeps arrestin in a basal conformation and suppresses an untimely mobilization of the secondary binding site (Gurevich (1998) *J. Biol. Chem.* 273:15501-15506). First, the ability of these mutants to
5 interact with purified β_2 AR reconstituted into phospholipid vesicles was tested by performing direct binding studies (Gurevich *et al.* (1995) *supra*) and agonist affinity shift assays (Gurevich *et al.* (1997) *supra*) *in vitro*. Wild type β -arrestin and β -arrestin-(1-393) bind poorly to activated unphosphorylated receptor (Fig. 1A). In contrast, β -arrestin-(Arg169 → Glu) and β -arrestin-(1-382) demonstrate significantly higher binding to
10 activated unphosphorylated receptor (Fig. 1A). Wild type β -arrestin and all three mutants readily bind to activated and phosphorylated β_2 AR (Fig. 1A). Thus, β -arrestin-(Arg169 → Glu) and β -arrestin-(1-382) bind to activated β_2 AR in a phosphorylation-independent fashion.

It was recently reported that arrestin-receptor complex is similar to G protein-receptor complex in two respects: agonists have higher affinity for arrestin-receptor complex
15 than for receptor alone, and only a fraction of the receptors forms such a high agonist affinity complex (HAC) even at saturating concentrations of arrestin (Gurevich *et al.* (1997) *supra*). The maximum percentage of the receptor in HAC gives a good estimate of the propensity of a given arrestin protein to bind tightly to the receptor (*arrestin competency*) (Gurevich *et al.* (1997) *supra*)). Consistent with the direct binding data (Fig. 1A), β -arrestin-(Arg169 → Glu) and β -arrestin-(1-382) induced the formation of HAC by unphosphorylated
20 β_2 AR ($22 \pm 4\%$ in both cases) (Fig. 2A). In contrast, all forms of β -arrestin induced the formation of HAC by phosphorylated β_2 AR (P- β_2 AR) (Fig. 2B). The percentage of HAC formed by P- β_2 AR in the presence of saturating ($1 \mu\text{M}$) concentration of β -arrestin, β -arrestin-(Arg169 → Glu), β -arrestin-(1-382), and β -arrestin-(1-393) was 31 ± 6 , 52 ± 3 , 41 ± 3 , and $20 \pm 4\%$, respectively (Fig. 2B). In summary, in both *in vitro* assays β -arrestin-(Arg169 → Glu) and β -arrestin-(1-382) demonstrate constitutive activity (phosphorylation-independent receptor binding).

Examples 2-3: Screening Assays Utilizing GPCRs and Phosphorylation-Independent Arrestin Mutants

The present invention is based at least in part, on the discovery that that phosphorylation-independent arrestin mutants are capable of bind GPCRs in a manner independent of any requirement for phosphorylation. It is now understood that this feature of the phosphorylation-independent mutants of the present invention that makes them particularly suited for use in *in vitro* screening assays. Accordingly, the present invention features screening assays designed to take advantage of the fact that phosphorylation-independent arrestin mutants can bind receptor (*e.g.*, GPCRs) in a manner independent of cellular receptor kinases. Examples 2-3 describe assays that are directed to identify natural and surrogate agonists for orphan GPCRs using phosphorylation-independent mutant human β -arrestins.

Example 4: High Throughput Screening Assays (HTS Assays)

Radiolabelled *in vitro* translated mutant human β -arrestin 2 Arg170 \rightarrow Glu and mutant human β -arrestin (1-382) are prepared as follows: A mutation at the codon encoding Arg170 is introduced in the cDNA sequence of human β -arrestin 2 by polymerase chain reaction in a suitable β -arrestin construct resulting in a phosphorylation recognition domain mutant. In a similar fashion, a mutation at the codon encoding amino acid 382 of human β -arrestin 2 is introduced by polymerase chain reaction in a suitable β -arrestin construct encoding a stop codon (\rightarrow TAG) resulting in Asp383 \rightarrow Ter. The mutant constructs are then used for *in vitro* transcription and translation, as described herein. *In vitro* translated Arg 170 \rightarrow Glu or Asp383 \rightarrow Ter is then radiolabelled to a suitable specific activity. High specific activity (>500 Ci/mmol) labelled mutants are preferred for subsequent use in screening assays.

A membrane preparation is prepared from cells that overexpress an orphan GPCR. Suitable cells for overexpression of orphan GPCRs include Sf9 cells and/or other mammalian cells suitable for overexpression of GPCRs. An aliquot of membrane preparation is contacted with an aliquot of radiolabelled *in vitro* translated Arg 170 \rightarrow Glu or Asp383 \rightarrow Ter β -arrestin mutant and the resulting mixture is contacted with a an aliquot of test compounds derived from a small molecule library. Specific binding is detected

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following a period of incubation of reactants by washing unbound radiolabelled *in vitro* translated Arg 170 → Glu or Asp383 → Ter β -arrestin mutant from the well. Assay conditions (e.g., concentration of assay components, composition of assay buffer, temperature and time of incubation) are optimized such that a signal-to-noise ratio of > 10-fold is achieved. Washing procedures such as high-salt or urea treatment may be used to achieve the desired signal-to-noise ratios.

Equal aliquots of membrane prep containing the orphan GPCR are distributed in the wells of a multiwell reaction vessel as are equal aliquots of radiolabelled *in vitro* translated Arg 170 → Glu or Asp383 → Ter β -arrestin mutant. Multiple wells are then reacted with different aliquots of test compounds, each aliquot containing a distinct mixture of potential surrogate ligands.

Example 5: BIACORE Screening Assays

Unlabelled *in vitro* translated mutant human β -arrestin 2 Arg 170 → Glu and mutant human β -arrestin (1-382) are prepared as described in Example 4.

A membrane preparation is prepared from cells that overexpress an orphan GPCR. An aliquot of membrane preparation is immobilized on a sensor chip and the immobilized membrane prep is contacted with an aliquot of *in vitro* translated Arg 170 → Glu or Asp383 → Ter β -arrestin mutant. The resulting mixture is contacted with an aliquot of test compounds derived from a small molecule library. Specific binding is detected following a period of incubation of reactants by detecting a change in surface plasmon resonance (SPR) signals generated from the reaction before and after the addition of test compound. Assay conditions are optimized such that a signal-to-noise ratio of > 3-fold is achieved.

Equal aliquots of membrane prep containing the orphan GPCR are distributed on sensor chips and contacted with equal aliquots of *in vitro* translated Arg 170 → Glu or Asp383 → Ter β -arrestin mutant. Sensor chips are then reacted with different aliquots of test compounds, each aliquot containing a distinct mixture of potential surrogate ligands.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

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What is claimed:

1. A method of identifying a G protein-coupled receptor (GPCR) ligand comprising contacting a composition comprising the GPCR and a constitutively active
5 arrestin mutant with a test compound and determining the ability of the test compound to modulate binding of the arrestin mutant to the GPCR, wherein modulation of binding indicates that the test compound is a GPCR ligand.
2. The method of claim 1; wherein the GPCR is an orphan receptor.
10
3. The method of claim 2, wherein the ligand identified is a surrogate
ligand.
4. A method of identifying a G protein-coupled receptor (GPCR) antagonist
15 comprising contacting a composition comprising the GPCR, a phosphorylation-independent arrestin mutant and a GPCR agonist with a test compound and determining the ability of the test compound to modulate binding of the arrestin mutant to said GPCR, wherein modulation of binding indicates that the test compound is a GPCR
antagonist.
20
5. The method of claim 1 or 4, wherein the constitutively active arrestin
mutant is a phosphorylation-independent mutant.
6. The method of claim 1 or 4, wherein the constitutively active arrestin
25 mutant has a mutation in the phosphorylation recognition domain.
7. The method of claim 1 or 4, wherein the constitutively active arrestin
mutant has a truncation of the C-terminal regulatory domain.

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8. The method of claim 6, wherein the constitutively active arrestin mutant has a mutation in an amino acid corresponding to any one of Arg171, Arg175, Lys 167 and Lys176 of SEQ ID NO:4.

5 9. The method of claim 6, wherein the constitutively active arrestin mutant has a mutation in an amino acid corresponding to Arg175 of SEQ ID NO:4.

10. The method of claim 6, wherein the constitutively active arrestin mutant has the amino acid sequence of SEQ ID NO:7.

10

11. The method of claim 7, wherein the constitutively active arrestin mutant has the amino acid sequence of SEQ ID NO:8.

12. The method of claim 1 or 4, wherein the composition is a membrane
15 preparation.

13. The method of claim 4, wherein the GPCR is a β -adrenergic receptor.

14. The method of claim 4, wherein the GPCR is a visual receptor.
20

15. The method of claim 1 or 4, wherein the arrestin mutant is a visual arrestin mutant.

16. The method of claim 1 or 4, wherein the arrestin mutant is a β arrestin
25 mutant.

17. The method of claim 1 or 4, wherein the arrestin mutant is an arrestin C mutant.

18. The method of claim 1 or 4, wherein the composition further comprises a
30 second GPCR.

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19. The method of claim 1 or 4, wherein the composition further comprises a plurality of other GPCRs which differ from said GPCR.
- 5 20. The method of claim 1 or 4, wherein the test compound is in an aqueous solution.
21. The method of claim 1 or 4, wherein the test compound is in an organic solution.
- 10 22. The method of claim 1 or 4, wherein the test compound is in a mixture of a plurality of test compounds.
23. The method of claim 1 or 4, wherein said arrestin mutant is conjugated to
15 a detectable marker.
24. The method of claim 1 or 4, wherein the detectable marker is a radioisotopic label.
- 20 25. The method of claim 1 or 4, wherein the detectable marker is a colorometric label.
26. The method of claim 1 or 4, wherein the detectable marker is a reporter
25 protein.
27. The method of claim 1 or 4, wherein determining the ability of the test compound to modulate binding of the arrestin mutant to the GPCR comprises detecting a change in surface plasmon resonance
- 30 28. The method of claim 22, wherein the mixture is a small molecule library.

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29. A method of identifying a G protein-coupled receptor (GPCR) ligand comprising contacting a GPCR with a phosphorylation-independent arrestin mutant and a test compound and determining the ability of the test compound to modulate binding of the arrestin mutant to the GPCR, wherein modulation of binding indicates that the test
5 compound is a GPCR ligand.

30. A method of identifying a surrogate ligand for an orphan G protein-coupled receptor (GPCR) comprising contacting a membrane preparation comprising said orphan GPCR and a phosphorylation-independent arrestin mutant with a test
10 compound and determining the ability of the test compound to effect binding of said arrestin mutant to said orphan GPCR.

31. A phosphorylation-independent β arrestin mutant.

15 32. The mutant of claim 31, having a mutation in the phosphorylation recognition region.

33. The mutant of claim 31, having a truncated C-terminal regulatory region.

20 34. The mutant of claim 31, having a mutation in the phosphorylation recognition region and a truncated C-terminal regulatory domain.

35. The mutant of claim 32, having a mutation in an amino acid corresponding to any one of Arg171, Arg175, Lys 167 and Lys176 of SEQ ID NO:4.
25

36. The mutant of claim 35, having a mutation in an amino acid corresponding to Arg175 of SEQ ID NO:4.

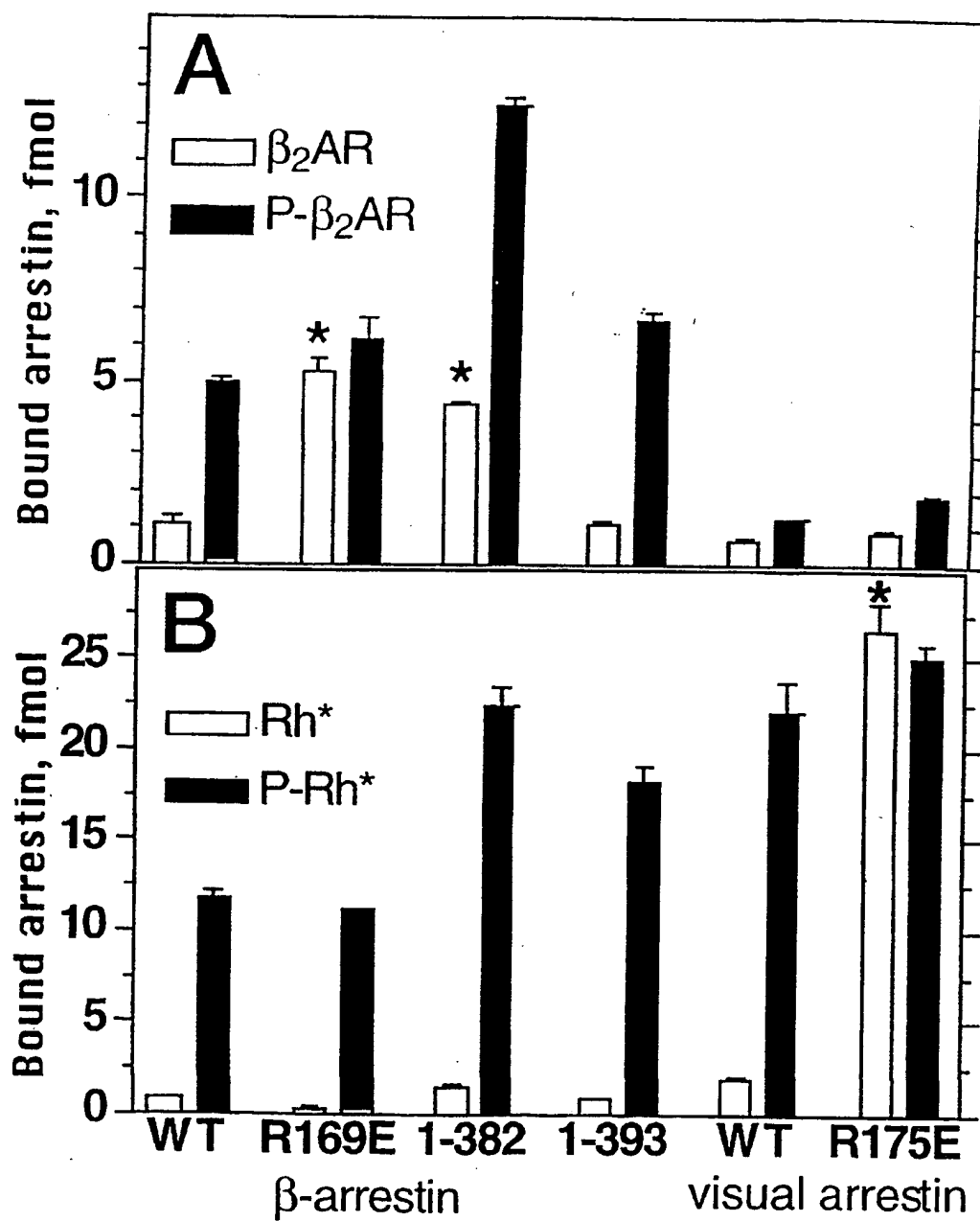
37. A phosphorylation-independent β arrestin mutant having the amino acid
30 of SEQ ID NO:7.

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38. A phosphorylation-independent β arrestin mutant having the amino acid sequence of SEQ ID NO:8.

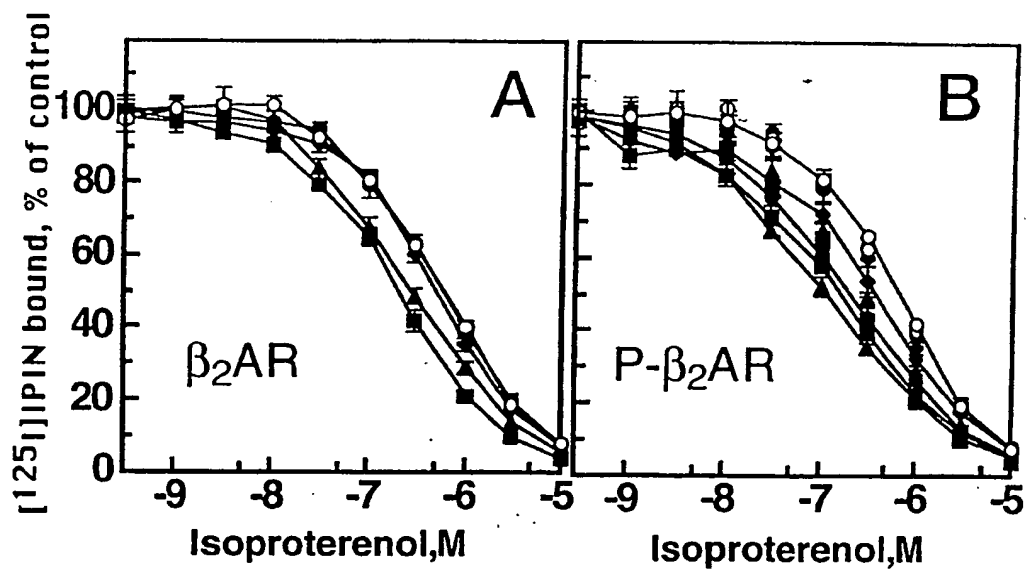
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FIG.1



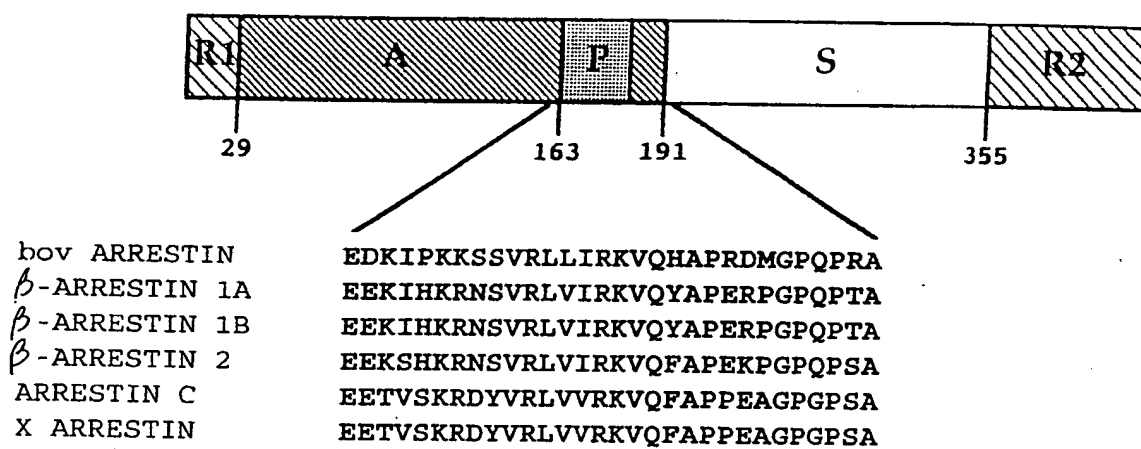
2/5

FIG.2



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FIG. 3



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FIG.4A

β -arrestin 1A	MGDK-GTRVFKKASPNGKLTVYLGKRDFVDHIDLVPDVGVLVDPEYLK
β -arrestin 1B	MGDK-GTRVFKKASPNGKLTVYLGKRDFVDHIDLVPDVGVLVDPEYLK
bov arrestin	MGDK-GTRVFKKASPNGKLTVYLGKRDFVDHIDLVEPVDGVLVDPEYLK
β -arrestin 2	MGEKPGTRVFKKSSPNCKLTVYLGKRDFVDHLDKVPDVGVLVDPDYLK
arrestin C	-----MSKVFKKTSSNGKLSIYLGKRDFVDHVDTVPEIDGVVLVDPEYLK
X arrestin	-----MSKVFKKTSSNGKLSIYLGKRDFVDHVDTVPEIDGVVLVDPEYLK
	::*****:*. * **::*****:*. * *:*****:***
β -arrestin 1A	ERRVYVTLTCAFRYGREDDVLGLTFRKDLFVANVQSFPAPEDKK-PLT
β -arrestin 1B	ERRVYVTLTCAFRYGREDDVLGLTFRKDLFVANVQSFPAPEDKK-PLT
bov arrestin	ERRVYVTLTCAFRYGREDDVLGLTFRKDLFVANVQSFPAPEDKK-PLT
β -arrestin 2	DRKVFTLTCAFRYGREDDVLGLSFRKDLFIATYQAFPPVPNPPR-PPT
arrestin C	CRKLFVMLTCAFRYGRDDLEVI GLTFRKDLYVQTLQVVPASSSPQGPLT
X arrestin	CRKLFVMLTCAFRYGRDDLEVI GLTFRKDLYVQTLQVVPASSSPQGALT
	::: *****:*. * *:*****:*. * *. . . : . *
β -arrestin 1A	RLQERLIKKLGEHAYPFTFEIPPNLPCSVTLQPGPEDTGKACGVDEYVKA
β -arrestin 1B	RLQERLIKKLGEHAYPFTFEIPPNLPCSVTLQPGPEDTGKACGVDEYVKA
bov arrestin	RLQERLIKKLGEHAYPFTFEIPPNLPCSVTLQPGPEDTGKACGVDEYVKA
β -arrestin 2	RLQDRLLRKLQGHAPFFFTIPQNLPCSVTLQPGPEDTGKACGVDFEIRA
arrestin C	VLQERLLHKLGDNAYPFTLQMVNLPCLSVTLQPGPEDAGKPCGIDFEVKS
X arrestin	VLQERLLHKLGDNAYPFTLQMVNLPCLSVTLQPGPEDAGKPCGIDFEVKS
	::***:*. * * : : *****:*. * *:***:***:
	*** * *
β -arrestin 1A	FCAENLEEKIHKRNSVRLVIRKVQYAPERPGPQPTAETTRQFLMSDKPLH
β -arrestin 1B	FCAENLEEKIHKRNSVRLVIRKVQYAPERPGPQPTAETTRQFLMSDKPLH
bov arrestin	FCAENLEEKIHKRNSVRLVIRKVQYAPERPGPQPTAETTRQFLMSDKPLH
β -arrestin 2	FCAKSLEEKSHKNSVRLVIRKVQFAPEKPGPQPSAETTRHFLMSDRSLH
arrestin C	FCAENPEETVSKRDYVRLVVRKVQFAPPEAGPGPSAQTIRRFLLSAQPLQ
X arrestin	FCAENPEETVSKRDYVRLVVRKVQFAPPEAGPGPSAQTIRRFLLSAQPLQ
	:*. * . ** : **:*. * * . . . * *:*. * *:***:*. * :
β -arrestin 1A	LEASLDKEIYYHGEPISVNVHVTNNTNKTVKKIKISVRQYADICLFNTAQ
β -arrestin 1B	LEASLDKEIYYHGEPISVNVHVTNNTNKTVKKIKISVRQYADICLFNTAQ
bov arrestin	LEASLDKEIYYHGEPISVNVHVTNNTNKTVKKIKISVRQYADICLFNTAQ
β -arrestin 2	LEASLDKELYHGEPLNVNVHVTNNTNKTVKKIKISVRQYADICLFNTAQ
arrestin C	LQAWMDREVHYHGEPISVNVSVNNCTNKKVKKIKISVDQITDVVLYSLDK
X arrestin	LQAWMDREVHYHGEPISVNVSVNNCTNKKVKKIKISVDQITDVVLYSLDK
	: *:***:***:*** :. * :. * :***:*** * *: * . :
β -arrestin 1A	YKCPVAMEEADDTVAPSSTFCKVYTLTPFLANNREKRLALDGKLKHEDT
β -arrestin 1B	YKCPVAMEEADDTVAPSSTFCKVYTLTPFLANNREKRLALDGKLKHEDT
bov arrestin	YKCPVAMEEADDTVAPSSTFCKVYTLTPFLANNREKRLALDGKLKHEDT
β -arrestin 2	YKCPVAQLEQDDQVSPSSTFCKVYTITPLSDNREKRLALDGKLKHEDT
arrestin C	YTKTVFIQEFTEETVAANSSFSQSFAVTPILAASCQKRLALDGKLKHEDT
X arrestin	YTKTVFIQEFTEETVAANSSFSQSFAVTPILAASCQKRLALDGKLKHEDT
	*. * * : *:. * :. :***:*. * :*****:*****

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FIG.4B

β-arrestin 1A	NLASSTLLREGANREILGIIVSYKVKVKLVESRGGLGDLASSDVAVELP
β-arrestin 1B	NLASSTLLREGANREILGIIVSYKVKVKLVESRG-----GDVAVELP
bov arrestin	NLASSTLLREGANREILGIIVSYKVKVKLVSRGGLGDLASSDVAVELP
β-arrestin 2	NLASSTIVKEGANKEVLGILVSYRVKVKLVVSRG-----GDVSVELP
arrestin C	NLASSTIIRPGMDKELLGILVSYKVRVNLMVSCGGILGDLTASDVGVELP
X arrestin	NLASSTIIRPGMDKELLGILVSYKVRVNLMVSCGGILGDLTASDVGVELP
	*****::: * ::*:***:***:*.***: * * .***.***
β-arrestin 1A	FTLMHPKPKEEP----PHREVPENETPVDTNLIELDTN---DDDIVFEDF
β-arrestin 1B	FTLMHPKPKEEP----PHREVPENETPVDTNLIELDTN---DDDIVFEDF
bov arrestin	FTLMHPKPKEEP----PHREVPEHETPVDTNLIELDTN---DDDIVFEDF
β-arrestin 2	FVLMHPKPHDHIPLRPQSAAPETDVPVDTNLIEFDTNYATDDDIVFEDF
arrestin C	LVLIHPKP-----SHEAAS-----SEDIVIEEF
X arrestin	LVLIHPKP-----SHEAAS-----SEDIVIEEF
	::*:***: .: .. .:***:***
β-arrestin 1A	AR-QRLKGMKDDKEEEDGTGSPQLNNR
β-arrestin 1B	AR-QRLKGMKDDKEEEDGTGSPQLNNR
bov arrestin	AR-QRLKGMKDDKEEEDGTGSPRLNDR
β-arrestin 2	AR-LRLKGMKDDDYDDQLC-----
arrestin C	TRKGEEESQKAVEAEGDEGS-----
X arrestin	TRKGEEESQKAVEAEGDEGS-----
	:* .: . * .: :

- 1 -

SEQUENCE LISTING

SEQ ID NO:1

MGDKGTRVFKKASPNGKLTVYLGKRDFVDHIDLVEPVDGVVLVDPEYLKERRVYVTLTCAFRYGREDLDV
 LGLTFRKDLFVANVQSFPAPEDKKPLTRLQERLIKKLGEHAYPFTFEIPPNLPCSVTLQPGPEDTGKAC
 GVDYEVKAFCAENLEEKIHKRNSVRLVIRKVQYAPERPGPQPTAETTRQFLMSDKPLHLEASLDKEIYYH
 GEPISVNVHVTNNTNKT VKKIKISVRQYADICLFNTAQYKCPVAMEEADDTVAPSSFTCKVYTLTPFLAN
 NREKRGLALDGLKHEDTNLASSTLLREGANREILGIIVSYKVKVLVVSRGGLLDGLASSDVAVELPFT
 LMHPKPKEEPPHREVPEHETPVDTNLIELDTNDDDIVFEDFARQRLKGMKDDKEEEDGTGSPRLNDR

SEQ ID NO:2

MGDKGTRVFKKASPNGKLTVYLGKRDFVDHIDLVPDVGVLVDPEYLKERRVYVTLTCAFRYGREDLDV
 LGLTFRKDLFVANVQSFPAPEDKKPLTRLQERLIKKLGEHAYPFTFEIPPNLPCSVTLQPGPEDTGKAC
 GVDYEVKAFCAENLEEKIHKRNSVRLVIRKVQYAPERPGPQPTAETTRQFLMSDKPLHLEASLDKEIYYH
 GEPISVNVHVTNNTNKT VKKIKISVRQYADICLFNTAQYKCPVAMEEADDTVAPSSFTCKVYTLTPFLAN
 NREKRGLALDGLKHEDTNLASSTLLREGANREILGIIVSYKVKVLVESRGGLLDGLASSDVAVELPFT
 LMHPKPKEEPPHREVPEHETPVDTNLIELDTNDDDIVFEDFARQRLKGMKDDKEEEDGTGSPQLNNR

SEQ ID NO:3

MGDKGTRVFKKASPNGKLTVYLGKRDFVDHIDLVPDVGVLVDPEYLKERRVYVTLTCAFRYGREDLDV
 LGLTFRKDLFVANVQSFPAPEDKKPLTRLQERLIKKLGEHAYPFTFEIPPNLPCSVTLQPGPEDTGKAC
 GVDYEVKAFCAENLEEKIHKRNSVRLVIRKVQYAPERPGPQPTAETTRQFLMSDKPLHLEASLDKEIYYH
 GEPISVNVHVTNNTNKT VKKIKISVRQYADICLFNTAQYKCPVAMEEADDTVAPSSFTCKVYTLTPFLAN
 NREKRGLALDGLKHEDTNLASSTLLREGANREILGIIVSYKVKVLVESRGGDVAVELPFTLMHPKPKE
 EPPHREVPEHETPVDTNLIELDTNDDDIVFEDFARQRLKGMKDDKEEEDGTGSPQLNNR

SEQ ID NO:4

MGEKPGTRVFKKSSPNCKLTVYLGKRDFVDHLDKVDVPDGVVLVDPDYLDKDRKVFVTLTCAFRYGREDLD
 VLGLSFRKDLFIATYQAFPPVPNPPRPTRLQDRLLRKLQHAHPFFFTIPQNLPCSVTLQPGPEDTGKA
 CGVDFEIRAFCAKSLEEKSHKNSVRLVIRKVQFAPEKPGPQPSAETTRHFLMSDRSLHLEASLDKELY
 HGEPLNVNVHVTNNTNKT VKKIKISVRQYADICLFSTAQYKCPVAQLEQDDQVSPSSFTCKVYTTPLLS
 DNREKRGLALDGLKHEDTNLASSTIVKEGANKEVLGILVSYRVKVKLVVSRGGDVSVELPFVLMHPKPH
 DHIPLPRPQSAAPETDVPVDTNLIEFDTNATDDDIVFEDFARLRLKGMKDDDYDDQLC

SEQ ID NO:5

MSKVFKKTSSNGKLSIYLGKRDFVDHVDTVEPIDGVVLVDPEYLKCRKLFVMLTCAFRYGRDDLEVIGLT
 FRKDLYVQTLQVVPASSSPQGPLTVLQERLLHKLGDNAYPFTLQMVNTNLPCSVTLQPGPEDAGKPCGID
 FEVKSFCANPEETVSKRDYVRLVVRKVQFAPPEAGPGPSAQTIRRFLLSAQPLQLQAWMDREVHYHGEPI
 ISVNVSVNNCTNKVIKKIKISVDQITDVVLYSLDKYTKTVFIQEFTETVAANSSFSQSFAVTPILAASCQ
 KRGLALDGLKHEDTNLASSTIIRPGMDKELLGILVSYKVRVNLVSCGILGDLTASDVGVELPLVLIIH
 PKPSHEAASSEDIVIEEFTTRKGEESQKAVEAEGDEGS

SEQ ID NO:6

MSKVFKKTSSNGKLSIYLGKRDFVDHVDTVEPIDGVVLVDPEYLKCRKLFVMLTCAFRYGRDDLEVIGLT
 FRKDLYVQTLQVVPASSSPQALTVLQERLLHKLGDNAYPFTLQMVNTNLPCSVTLQPGPEDAGKPCGID

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FEVKSFCAENPEETVSKRDYVRLVVRKVQFAPPEAGPGPSAQTIRRFLLSAQPLQLQAWMDREVHYHGEP
ISVNVSIINNCTNKVIKKIKISVDQITDVVLYSLDKYTKTVFIOEFTETVAANSSFSQSFAVTPILAASCQ
KRGALDGLKHKHEDTNLASSTIIRPGMDKELLGILVSYKVRVNLVSVSCGGILGDLTASDVGVLPVLVLIH
PKPSHEAASSEDIVIEEFTRKGEEESQKAVEAEGDEGS

SEQ ID NO:7

MGEKPGTRVFKKSSPNCKLTVYLGKRDFVDHLDKVDVPDGVVLVDPDYLKDRKVFVTLTCAFRYGREDLD
VLGLSFRKDLFIATYQAFPPVNP RPPTRLQDRLLRKLQHAHPFFFTIPQNLPCSVTLQPGPEDTGKA
CGVDFEIRAFCAKSLEEKSHKRNSVRLVIEKVQFAPEKPGPQPSAETTRHFLMSDRSLHLEASLDKELY
HGEPLNVNVHVTNNSTKTVKKIKVSVRQYADICLFSTAQYKCPVAQLEQDDQVSPSSTFCKVYTITPLLS
DNREKRGALDGLKHKHEDTNLASSTIVKEGANKEVLGILVSYRVKVLVSVRGGDVSVELPFVLMHPKPH
DHIPLRPQSAAPETDVPVDTNLIEFDTNYATDDDIVFEDFARLRLKGMKDDDYDDQLC

SEQ ID NO:8

MGEKPGTRVFKKSSPNCKLTVYLGKRDFVDHLDKVDVPDGVVLVDPDYLKDRKVFVTLTCAFRYGREDLD
VLGLSFRKDLFIATYQAFPPVNP RPPTRLQDRLLRKLQHAHPFFFTIPQNLPCSVTLQPGPEDTGKA
CGVDFEIRAFCAKSLEEKSHKRNSVRLVIRKVQFAPEKPGPQPSAETTRHFLMSDRSLHLEASLDKELY
HGEPLNVNVHVTNNSTKTVKKIKVSVRQYADICLFSTAQYKCPVAQLEQDDQVSPSSTFCKVYTITPLLS
DNREKRGALDGLKHKHEDTNLASSTIVKEGANKEVLGILVSYRVKVLVSVRGGDVSVELPFVLMHPKPH
DHIPLRPQSAAPETDVPVDTNLIEFDTNYAT

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C07K 14/47

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LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX,
MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL,
TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

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patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,
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(15) Information about Correction:

Previous Correction:

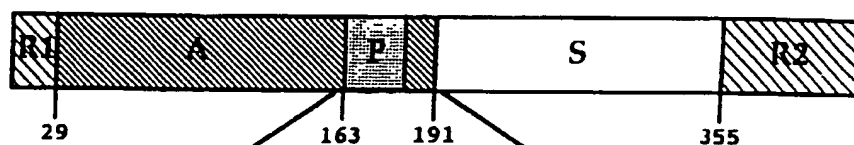
see PCT Gazette No. 07/2002 of 14 February 2002, Section
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(74) Agents: MANDRAGOURAS, Amy, E.; Lahive & Cock-
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*For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.*

(54) Title: IDENTIFICATION OF G PROTEIN-COUPLED RECEPTOR LIGANDS AND MODULATORS



hov ARRESTIN
 β -ARRESTIN 1A
 β -ARRESTIN 1B
 β -ARRESTIN 2
ARRESTIN C
X ARRESTIN

EDKIPKKSSVRLIRKVQHAPRDMGPQPRA
EEKIHKRNSVRLVIRKVQYAPERPGPQPTA
EEKIHKRNSVRLVIRKVQYAPERPGPQPTA
EEKSHKRNSVRLVIRKVQFAPEKPGPQPSA
EETVSKRDYVRLVVRKVQFAPPEAGPGPSA
EETVSKRDYVRLVVRKVQFAPPEAGPGPSA

(57) Abstract: The present invention features methods of assaying for ligands and/or modulators of G protein-coupled receptors, for example, orphan G protein-coupled receptors. The methods of the invention feature constitutively active arrestin mutants, in particular, phosphorylation independent mutants. Also described are specific phosphorylation independent arrestin mutants and methods of making such mutants.

WO 01/67106 A3

INT/ NATIONAL SEARCH REPORT

International Application No

PCT/US 01/07304

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 G01N33/566 C07K14/47

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 G01N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, MEDLINE, EMBL, PAJ, WPI Data, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KOVOOR ABRAHAM ET AL: "Targeted construction of phosphorylation-independent beta-arrestin mutants with constitutive activity in cells." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 274, no. 11, 12 March 1999 (1999-03-12), pages 6831-6834, XP002187872 ISSN: 0021-9258 cited in the application the whole document	1,4-14, 16,18, 29,31-38
Y		30
A		2,3,15, 17,25-27
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☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

* Special categories of cited documents:

A document defining the general state of the art which is not considered to be of particular relevance

E earlier document but published on or after the international filing date

L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

G document member of the same patent family

Date of the actual completion of the international search

23 January 2002

Date of mailing of the international search report

06/02/2002

Name and mailing address of the ISA

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Fax (+31-70) 340-3016

Authorized officer

Tuyman, A

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 01/07304

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p> GUREVICH VSEVOLOD V ET AL: "Visual arrestin binding to rhodopsin: Diverse functional roles of positively charged residues within the phosphorylation-recognition region of arrestin." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, no. 11, 1995, pages 6010-6016, XP002187873 ISSN: 0021-9258 cited in the application the whole document figures 1-5 </p>	31-36
A		1,4-9, 15,17
A	<p> BIERI CHRISTOPH ET AL: "Micropatterned immobilization of a G protein-coupled receptor and direct detection of G protein activation" NATURE BIOTECHNOLOGY, NATURE PUBLISHING, US, vol. 17, no. 11, November 1999 (1999-11), pages 1105-1108, XP002183971 ISSN: 1087-0156 abstract </p>	1,4,27
Y	<p> BARAK L S ET AL: "A beta-arrestin/green fluorescent protein biosensor for detecting G protein-coupled receptor activation" JOURNAL OF BIOLOGICAL CHEMISTRY, AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD, US, vol. 44, no. 272, 31 October 1997 (1997-10-31), pages 27497-27500, XP002076354 ISSN: 0021-9258 abstract </p>	30
A		1-4,25, 26

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Present claims 31-38 relate to a product defined by reference to a desirable characteristic or property, namely a phosphorylation-independent beta arrestin mutant.

The claims cover all products having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such products. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the product by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the products mentioned in the description on page 8 lines 12-31. From this description and the sequence of SEQ ID NO 4, it is clear that the mutations mentioned in claim 35 and 36 are clerical mistakes. The beta-arrestin 2 mutants of SEQ ID NO 4 that have been searched, have according to page 8, line 15 a mutation in an amino acid corresponding to Lys 161, Arg 162, Arg 166, Arg 170 and Lys 171.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

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HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK,
LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX,
MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL,
TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

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patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,
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